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Mutated Epitopes of Hepatitis B Surface Antigen Fused to the Core Antigen of the Virus Induce Antibodies That React With the Native Surface Antigen

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Fusion of peptide epitopes to the core antigen (HBcAg) of hepatitis B virus (HBV) enhances their immunogenicity, both quantitatively and qualitatively. In a number of vaccine-induced mutants of HBV, glycine$_{145}$ of the surface antigen S polypeptide (HBsAg) has been replaced by arginine, resulting in loss of cross-reactivity with antibodies to normal (wild-type) HBsAg. HBcAg fusion proteins carrying the immunodominant epitope of HBsAg, in which glycine$_{145}$ was replaced by arginine, glutamic acid, or lysine, were produced in *Escherichia coli* and formed particles that displayed HBc antigenicity and immunogenicity similar to that of HBcAg itself. The fusion proteins also elicited T-cell-proliferative responsiveness to HBcAg and HBsAg. Fusions carrying either wild-type or mutated epitopes of HBsAg showed HBs antigenicity in immunoblot analysis and antigen-capture immunoradiometric assay, but both mutant and wild-type derivatives induced antibodies that cross-reacted with wild-type HBsAg. The results emphasise the potential for HBcAg fusion proteins in vaccines by broadening the antibody response in a way that could confer protection against both wild-type and variant forms of HBV. ***J. Med. Virol. 51:159–166, 1997.***

KEY WORDS: hepatitis B; fusion protein; vaccine; mutant antigen; serological specificity

INTRODUCTION

The nucleocapsid protein or core antigen (HBcAg) of hepatitis B virus (HBV) can be produced efficiently in *Escherichia coli* [Burrell et all, 1979; Stahl et al., 1982], giving a product closely resembling the particles found in the liver of infected individuals [Cohen and Richmond, 1982; Crowther et al., 1994]. Fusion of peptide sequences to HBcAg enhances their immunogenicity, probably because the HBcAg component provides T-cell help [Milich et al., 1987a], and the fusion proteins retain the polymeric or particulate nature of HBcAg [Stahl and Murray, 1989]. Peptide sequences of varying length have been fused to HBcAg at the N-terminus [Stahl et al., 1982; Clarke et al., 1987], the truncated C-terminus [Stahl and Murray, 1989; Schodel et al., 1992; Borisova et al., 1989; Yoshikawa et al., 1993], or some internal sites [Schodel et al., 1992; Francis, 1991; Borisova et al., 1993] without loss of the self-assembly properties of HBcAg. Fusions of sequences carrying the immunodominant region of the surface antigen (HBsAg; amino acids [aa] 111–156 or 111–165) to the C-terminus of truncated HBcAg gave the proteins HBcS$_{(111–156)}$ and HBcS$_{(111–165)}$, which were produced in *E. coli* to give particulate products that induced an anti-HBs response [Stahl and Murray, 1989].

Immune responses to HBsAg are complex. In addition to epitopes residing in the preS1 and preS2 domains [Heermann et al., 1984] of the minor components (the long, L, and medium, M, polypeptides) of HBsAg, a major dominant type-specific epitope, α, and a number of variable subtype determinants have been assigned to the more abundant component of HBsAg (the short, S, polypeptide) on the basis of serological studies [Le Bouvier, 1971; Bancroft et al., 1972; Courroucé-Pauty and Holland, 1978]. Monoclonal antibodies exhibiting different specificities have been obtained more recently against HBsAg of various serotypes [Usuda et al., 1986; Waters et al., 1992a]. Although the HBsAg coding sequence determined on viral DNA cloned from sera of differing subtypes shows differences in the corresponding protein sequences, site-specific mutations of the apparently critical residues did not effect a definitive change from one serological subtype (*y*) to another (*d*) but produced a gradual change, with both *y* and *d* reactivities and immunogenicities being manifest from the same molecule [Ashton-Rickardt and Murray, 1989]. The mutations con-

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cerned were made within or close to the major immunodominant region of HBsAg, which appears to be very sensitive to conformation. They were all within the segment used for construction of the HBcS fusion proteins, raising the possibility that such fusion proteins may provide a means for changing the specificity of the immune response to the additional epitopes.

The emergence of an escape mutant with arginine substituted for glycine at residue 145 of HBsAg [Carman et al., 1990], the single substitution in the molecule being sufficient to abrogate cross-reactivity with anti-HBs [Bruce and Murray, 1995], provided the impetus for this study, with HBcAg fusions carrying the immunodominant region of the S protein, $HBs_{(111–156)}$, with the mutations $Gly_{145 \rightarrow Arg}$ (to mimic the escape mutant), $Gly_{145 \rightarrow Glu}$, and $Gly_{145 \rightarrow Lys}$. The humoral and cellular immune responses to these fusion proteins were studied in rabbits and showed that antibodies to the Arg_{145} mutant could cross-react with the wild-type HBsAg.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

E. coli strains RB791 [Amnan et al., 1983], BMH 71-18 *mutL* [Kramer et al., 1984], and TG1 and plasmids $pHBcS_{(111–156)}$, $pHBcPreS_{1(1–36)}$, and $pHBcPreS_2$ [Stahl and Murray, 1989] were used.

Cloning Reagents and Techniques

Plasmid DNA was manipulated essentially as described elsewhere [Sambrook et al., 1989]. Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim (Mannheim, Germany) and DNA polymerase I (Klenow fragment) from Northumbria Biologicals Ltd (NBL, Cramlington, UK). Oligonucleotides were obtained from the Oswel DNA Unit, Department of Chemistry, University of Edinburgh. DNA sequences were determined using the chain-termination method [Sanger et al., 1977].

Construction of Mutant $pHBcS_{(111–156)}$ Plasmids

The target for mutagenesis, a 594-bp EcoRI-SalI fragment bearing the $HBc_{(3–144)}$ and $HBs_{(111–156)}$ sequences, was excised from $pHBcS_{(111–156)}$ and subcloned into the M13mp19 vector. Site-directed mutagenesis was carried out by the double-primer method [Zoller and Smith, 1987] to convert the codon for aa residue 145 of HBsAg from Gly to Arg, Glu, or Lys. The respective oligonucleotides used were 412G (5′-ACC TTC GGA TAG AAA CTG CAC CT-3′), 411G (5′-ACC TTC GGA TGA AAA CTG CAC CT-3′), and 359G (5′-AAA CCT TCG GAT AAA AAC TGC ACC TG-3′). *E. coli* BMH71-18 *mutL* was used for transfection, with *E. coli* TG1 providing the lawn. The M13mp19 clones containing a mutated insert were identified by sequencing. The entire insert of the candidate mutants was fully sequenced.

The 594-bp EcoRI-SalI fragment from each mutant M13mp19 derivative was transferred to plasmid pHBcX [Rossner, 1991], a derivative of ptacHpaIIR2 [Stahl and Murray, 1989] which had been linearised by digestion with restriction enzymes EcoRI and SalI to give the recombinant plasmids $pHBcS_{145R}$, $pHBcS_{145E}$, and $pHBcS_{145K}$, from which the respective HBc fusion proteins were expressed from the inducible *tac* promoter.

Purification of HBcAg Fusion Proteins Produced in *E. coli*

The HBcAg fusion proteins were expressed in transformants of *E. coli* strain RB791, which were grown, induced, harvested, and lysed as described previously [Stahl and Murray, 1989]. Cell debris was removed by centrifugation and protein precipitated from the supernatant by adding ammonium sulphate to 30% saturation. The precipitate was resuspended in 50 mM Tris-HCl, pH 8.0, and dialyzed against the same buffer and the fusion protein particles collected by centrifugation (100,000 *g* for 1 hr); resuspended in 50 mM Tris-HCl, pH 8.0; and chromatographed on Sepharose 4B-Cl in the same solvent containing 0.2 mM phenylmethylsulphonyl fluoride and 5 μM NaN_3. Fractions containing the fusion proteins (identified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis [SDS-PAGE]) were pooled and the product collected by centrifugation (100,000 *g,* 1 hr), and pellets were suspended in phosphate-buffered saline (PBS) and dialyzed against the same buffer.

HBc and HBs Antigenicity

HBcAg reactivity was determined by antigen-capture immunoradiometric assay (IRMA). Polystyrene beads (6.4 mm o.d., specular finish; NBL) coated with human anti-HBc IgG were incubated with test samples overnight at room temperature, washed, and incubated with [^{125}I]-labelled human anti-HBc IgG at 45°C for 2 hr. Beads were then washed and their radioactivity counted. HBs antigenicity was determined by both the antigen-capture IRMA using two antibodies with different specificity and the AUSRIA II-125 diagnostic kit (Abbott Laboratories, North Chicago, IL), which was used as recommended by the manufacturer. In the IRMA method, the HBcAg fusion proteins were captured on polystyrene beads coated with human anti-HBc IgG and HBsAg reactivity was detected by incubation with rabbit anti-HBs antibody followed by [^{125}I]-labelled donkey anti-rabbit IgG. HBsAg and IgG preparations were labelled with ^{125}I using the iodogen-based method [Burrell, 1975; Burrell et al., 1978].

Immunization

Six-month old outbred Dutch male rabbits were immunized i.m. with 0.2 or 1 mg of HBcAg fusion proteins emulsified in Freund's complete adjuvant and subsequently boosted a number of times with the same antigen emulsified in Freund's incomplete adjuvant.

Lymphocyte Proliferation Assay

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation through density gradients of Ficoll-Paque (Pharmacia, Uppsala, Sweden). PBMC (2×10^5 or 5×10^5) in 0.2 ml of medium (RPMI 1640 medium supplemented with 2 mM L-Gln, 10 mM

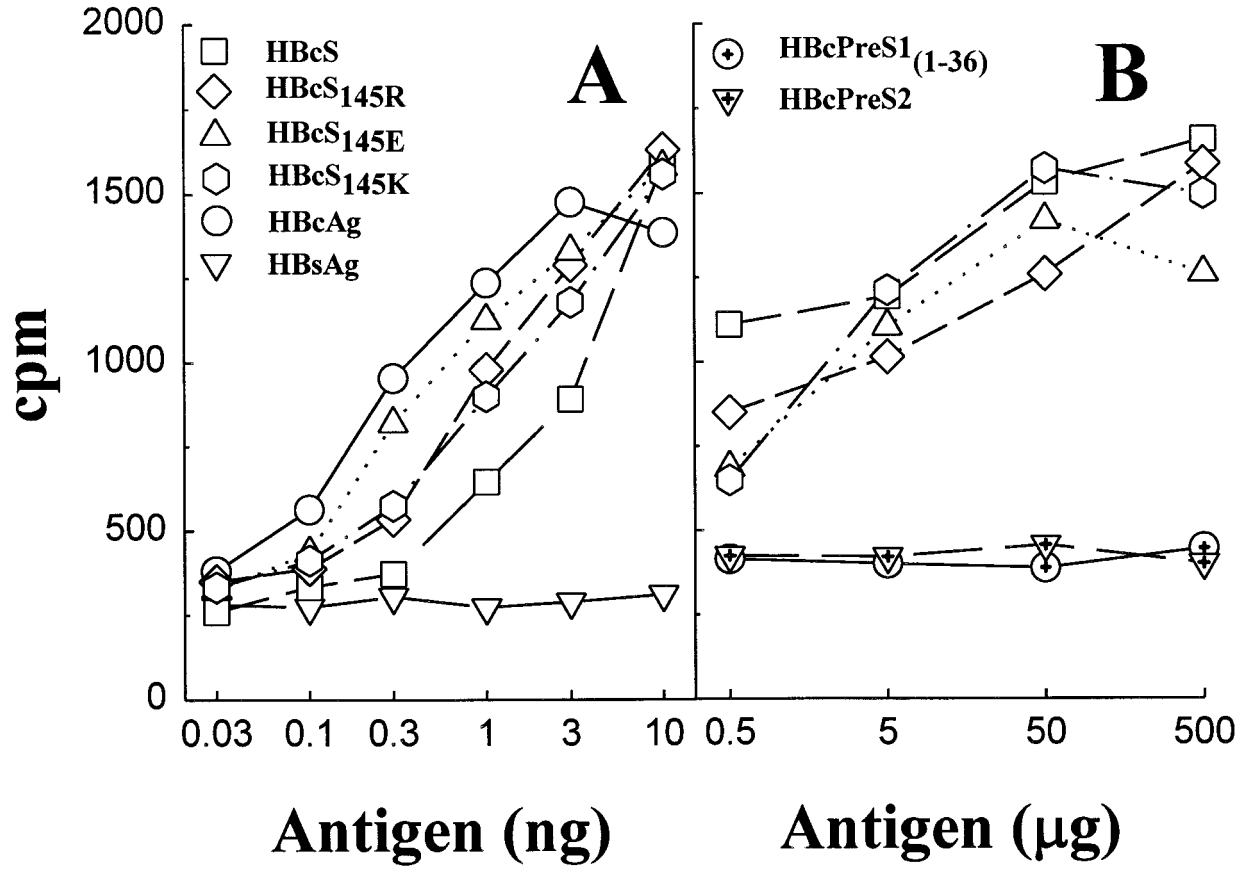

Fig. 1. Antigenic reactivities of HBcAg and the HBcS fusion proteins measured by antigen-capture IRMA on beads coated with anti-HBc IgG. **A:** HBcAg reactivity of bound antigens measured by capture of [^{125}I]-labelled anti-HBc IgG. **B:** HBsAg reactivity of bound antigens measured by capture of rabbit anti-HBs serum followed by [^{125}I]-labelled donkey anti-rabbit IgG.

Hepes, 5 × 10^{-5} M 2-mercaptoethanol, 10 mM $NaHCO_3$, and 5% fetal calf serum) were incubated for 5 days at 37°C in a humidified CO_2 atmosphere alone or with various concentrations of recombinant HBcAg (Biogen, Cambridge, MA), recombinant HBsAg (Biogen or Green Cross, Osaka, Japan), or HBcAg fusion proteins. The proliferative potential and nonspecific T-cell activation of lymphocytes were monitored with concanavalin A (Pharmacia; 2 μg/ml) and poly (Na glutamate, alanine, tyrosine; 6:3:1, m.w. 20,000–50,000, 5 μg/ml; Sigma, St. Louis, MO), respectively. During the final 16 hr, [^{3}H]thymidine (5 Ci/mmole; Amersham, Aylesbury, UK; 1 μCi) was added. Cells were harvested on filter strips for determination of [^{3}H]dT incorporation; data are expressed as the mean of the cpm ± the deviation from the mean. The stimulation index is the ratio of [^{3}H]-dT incorporation into stimulated cells to that into nonstimulated cells.

Assay of Anti-HBc and Anti-HBs Antibodies

A competitive radioimmunoassay (RIA) was used to detect anti-HBc [Murray et al., 1984]; the titre is expressed as the reciprocal of the serum dilution giving 50% inhibition of the binding of [^{125}I]-labelled anti-HBc to HBcAg on a solid phase. Anti-HBs antibody was detected by the AUSAB diagnostic kit (Abbott Laboratories), which was used according to the manufacturer's instructions, or, in liquid phase, by the double antibody radioimmunoprecipitation (DARIP) test [Burrell et al., 1978]. In the DARIP assay, 10-fold dilutions of the test sera were incubated with [^{125}I]-HBsAg (*adw* or *ayw* subtype), followed by addition of donkey anti-rabbit IgG to precipitate antigen-antibody complexes. The amount of anti-HBs antibody in the test serum is expressed as the mean of the percentage of [^{125}I]-HBsAg coprecipitated from duplicate test samples.

RESULTS

Purification and Properties of HBcAg Fusion Proteins

Fusion proteins were produced in *E. coli* in yields ranging from 5 to 34 mg/l of culture. All fusion proteins resembled HBcAg in giving strongly positive reactions with polyclonal anti-HBc antiserum in immunoblot analysis, and electron-microscopic analysis of the fusion proteins carrying the wild-type or mutated $HBs_{(111–156)}$ segment showed that they all assembled into particles morphologically similar to HBcAg itself. In antigen-capture assays (IRMA), all HBcAg fusion proteins displayed similar HBcAg reactivity and their binding to anti-HBc antibody showed a dose-response similar to that of full-length HBcAg (Fig. 1A).

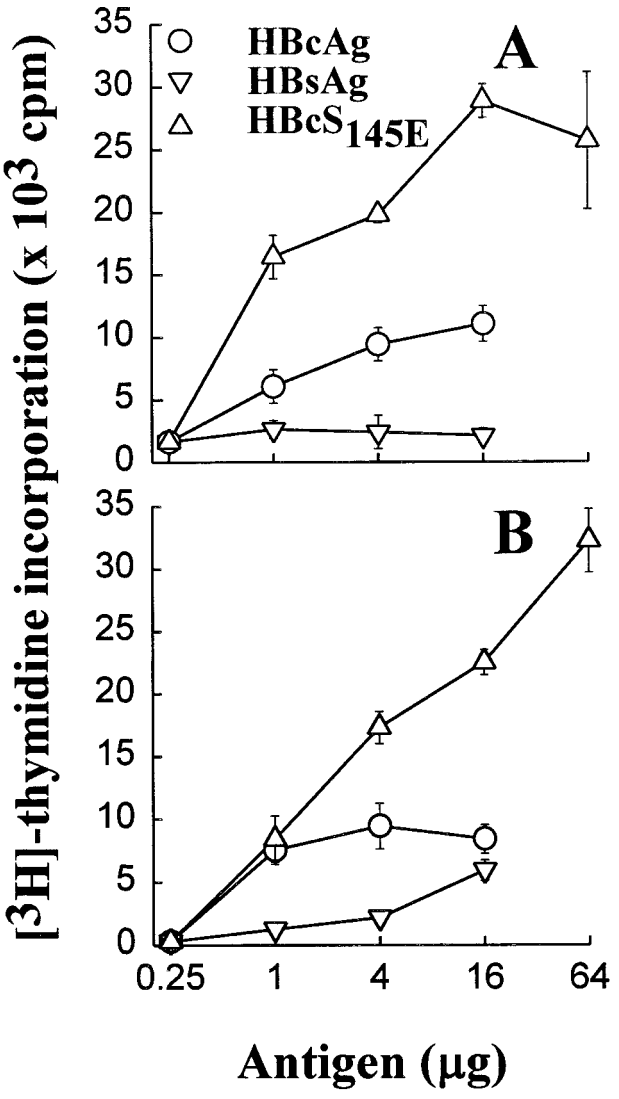

Fig. 2. Lymphocyte-proliferative responses to PBMC taken from a rabbit (677) **(A)** on day 13 and **(B)** on day 93 (13 days after a second boost) after immunization with $HBcS_{145E}$. PBMC were cultured with HBcAg, HBsAg, or $HBcS_{145E}$ at different concentrations for 5 days. Cultures were pulse-labelled with [^{3}H]dT 16 hr before harvest and incorporation determined by liquid scintillation counting. Results are expressed as the mean of the cpm incorporated in triplicate or quadruplicate cultures ± the deviation from the mean.

None of the fusion proteins displayed significant HBsAg reactivity in the AUSRIA II-125 test, but HBcS and its three mutant counterparts all gave a positive reaction with antibodies against denatured or native HBsAg in immunoblot analyses (data not shown), indicating that the HBs sequence was presented in recognisable form in the HBcAg fusion proteins. With high concentrations of the HBcAg fusion proteins carrying a wild-type or mutant $HBs_{(111–156)}$ segment, HBs antigenicity was detectable by antigen-capture IRMA using two antibodies with different specificity, whereas HBcAg, $HBcPreS_{1(1–36)}$, and $HBcPreS_2$ showed no HBs antigenicity in this assay (Fig. 1B).

T-Cell Responses

Dose-response curves show that proliferation of PBMC taken from a rabbit (677) 13 days after immunization (Fig. 2A) and 13 days after a second booster inoculation (day 93; Fig. 2B) with $HBcS_{145E}$ was stimulated by HBcAg and, more potently, by $HBcS_{145E}$. However, the PBMC taken on day 13 did not respond to stimulation with HBsAg, but those taken on day 93 did, albeit to a much lesser extent than to the fusion protein or HBcAg. There was no in vitro stimulation of PBMC taken before immunization with $HBcS_{145E}$, HBcAg, or HBsAg.

Figure 3 summarizes the proliferation observed in response to optimal doses of the antigens with PBMC taken from rabbits at different times after immunization and booster inoculations with either HBcS or its Gly_{145} mutants. The magnitude of the response to HBcS fusion proteins, HBcAg, or HBsAg varied amongst the four animals. The lymphocyte-proliferative response to HBcS and its mutants and to HBcAg appeared in cells taken 7 days after immunization and was maintained, but at fluctuating levels, in cells taken throughout the experiment (93 days). In most cases, the proliferative responses to the fusion proteins and to HBcAg were greatly enhanced after booster inoculations of the rabbits, where the responses had declined. Proliferative responses to HBsAg were generally very poor, with a weak and delayed response observed only in the rabbit injected with $HBcS_{145E}$ and only after booster inoculations.

Immunogenic Properties of HBcS Fusion Proteins

Figure 4 shows the production of anti-HBc antibody in four rabbits after immunization and subsequent boost with HBcS carrying the wild-type or Gly_{145} mutant $HBs_{(111–156)}$ segment. All elicited high responses, with titres reaching maximal levels 12–22 days after the second boost or 12 days after the third boost in one of the animals and remaining at high levels.

Although the HBsAg coding region in the HBcAg fusion constructions was derived from the same DNA sequence as that in pHinG2 which expresses HBsAg exhibiting *ayw* rather than *adw* specificity with respect to monospecific antisera [Ashton-Rickardt and Murray, 1989], anti-HBs antibody induced by the HBcS fusion proteins reacted equally effectively with [^{125}I]-labelled HBsAg of either *adw* or *ayw* subtypes in the DARIP assay (data not shown), indicating that these antibodies were directed primarily toward the *a* group-specific determinant on HBsAg. The anti-HBs responses induced by the various fusion proteins are shown in Figure 5. In the rabbit (675) immunized with HBcS, a low level of anti-HBs antibody was observed 21 days after immunization and increased following two booster inoculations, reaching a peak 14 days after the second boost (day 93). The animal (676) immunized with $HBcS_{145R}$, gave no detectable anti-HBs before day 21,

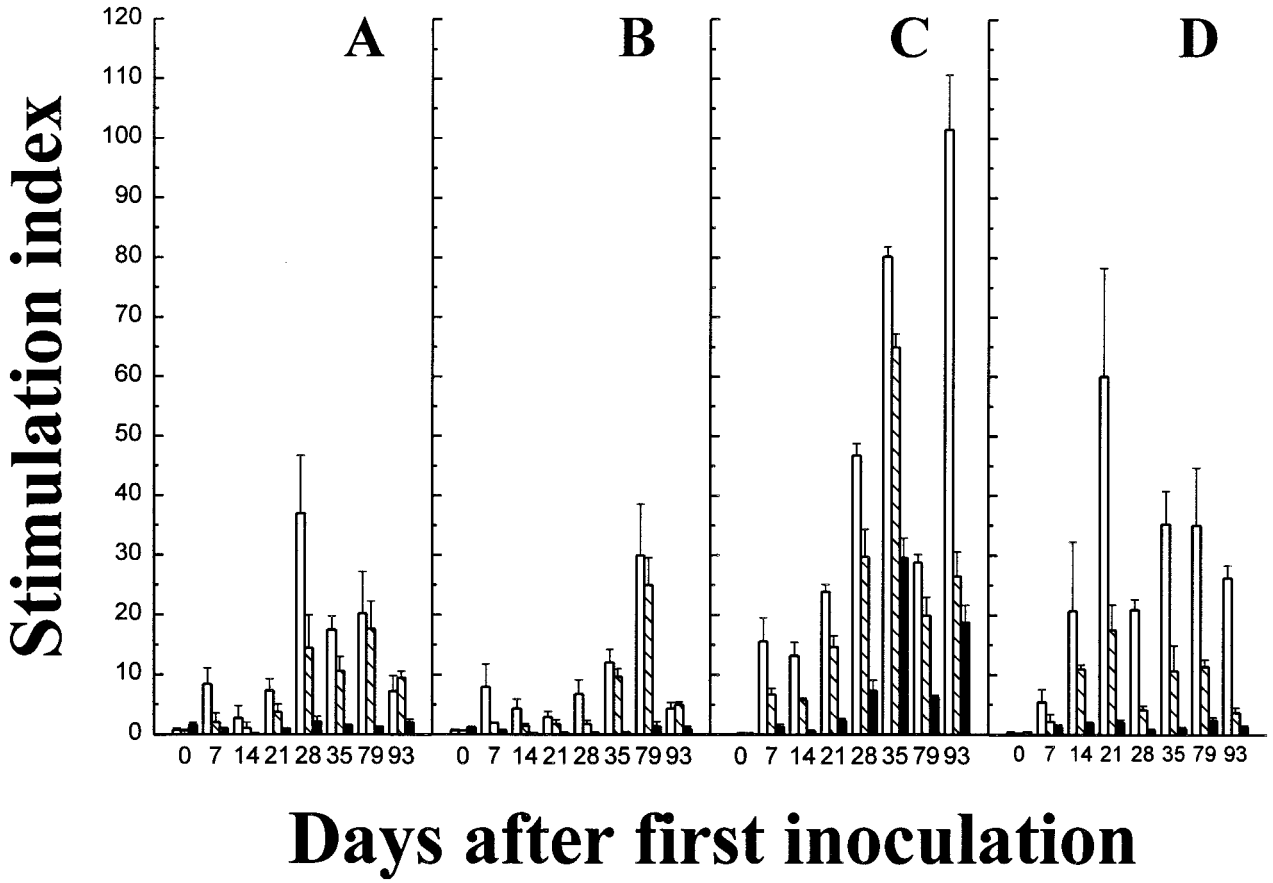

Fig. 3. Lymphocyte-proliferative responses in PBMC taken from four rabbits inoculated with **(A)** HBcS, **(B)** $HBcS_{145R}$, **(C)** $HBcS_{145E}$, and **(D)** $HBcS_{145K}$ on days 0, 21, and 81. After culture with the corresponding HBcAg fusion protein (64 μg/ml), HBcAg (16 μg/ml), or HBsAg (16 μg/ml) for 5 days, cells were pulse-labelled with [^{3}H]dT. The stimulation index was calculated as the ratio of [^{3}H]dT incorporated into stimulated cells to that incorporated into nonstimulated cells (mean ± deviation from the mean). HBcS (wild-type and mutants), open bars; HBcAg, hatched bars; HBsAg, solid bars.

but after boosting, the response was similar to that observed with HBcS, although at a lower level. Anti-HBs was not detectable in the rabbit (677) immunized with $HBcS_{145E}$ until 14 days after the second boost (day 93) and then at a much lower level than in rabbits 675 and 676. No anti-HBs was detectable in any of the samples from the rabbit (678) immunized with $HBcS_{145K}$, even though three booster inoculations were given.

Anti-HBs titres in some sera were also measured by the AUSAB test, which gave results in general agreement with those from the DARIP assay. Sera negative for anti-HBs antibody by the DARIP assay were also negative in the AUSAB test.

DISCUSSION

Apart from the higher immunogenicity of HBcAg in terms of T-cell activation and antibody production compared to HBsAg [Milich et al., 1987b], the wide range of hosts for its expression makes it an attractive carrier for presentation of foreign epitopes, particularly since its T-cell-independent nature may be exploitable to advantage for vaccine development for use in T-cell-compromised individuals [Milich and McLachlan, 1986]. Although anti-HBc antibody is not virus-neutralizing, inoculation with HBcAg has been shown to protect chimpanzees against HBV infection [Murray et al., 1984, 1987; Iwarson et al., 1985) probably because HBcAg-primed T cells can induce antibody production following exposure to other epitopes that are physically linked to HBcAg, such as HBsAg in HBV [Milich et al., 1987a; Murray, 1988). Further, HBcAg-specific Th cells can induce anti-S antibody production in mice that do not otherwise respond to HBsAg [Milich et al., 1987a].

In the work described here, HBcAg was exploited to present epitopes from the immunodominant region of HBsAg, $HBs_{(111–156)}$, carrying either wild-type or mutant HBsAg sequences. The fusion protein $HBcS_{(111–156)}$ did not display the typical antigenic reaction of HBsAg in a standard RIA or enzyme-linked immunosorbent assay (ELISA), but immunoblot analyses of HBcS and the three mutant counterparts with polyclonal antibodies against HBsAg showed that $HBs_{(111–156)}$ was presented on the hybrid HBcAg particles. This assay favours linear determinants, and the

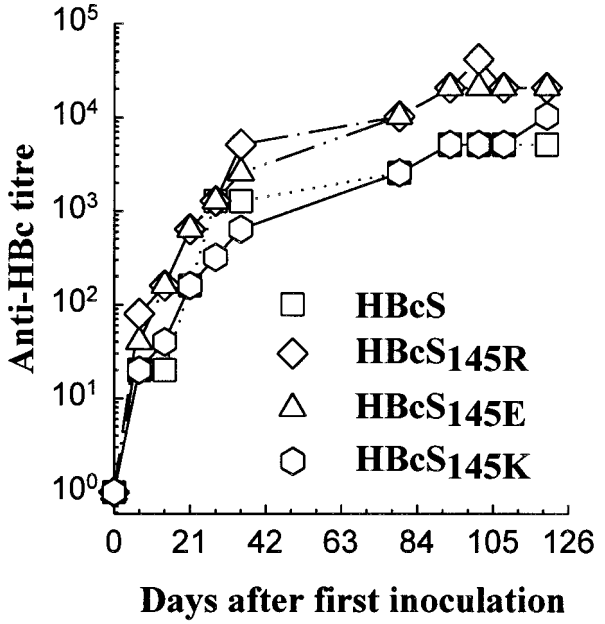

Fig. 4. Anti-HBc titres in four rabbits immunized with HBcS or its Gly_{145} mutants. Rabbits 675, 676, 677, and 678 were inoculated with HBcS, $HBcS_{145R}$, $HBcS_{145E}$, and $HBcS_{145K}$, respectively, on days 0, 21, 80, and 108. Serum samples were serially diluted and assayed for anti-HBc antibody by competitive RIA. Titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of [^{125}I]-labelled anti-HBc binding to HBcAg on a solid phase.

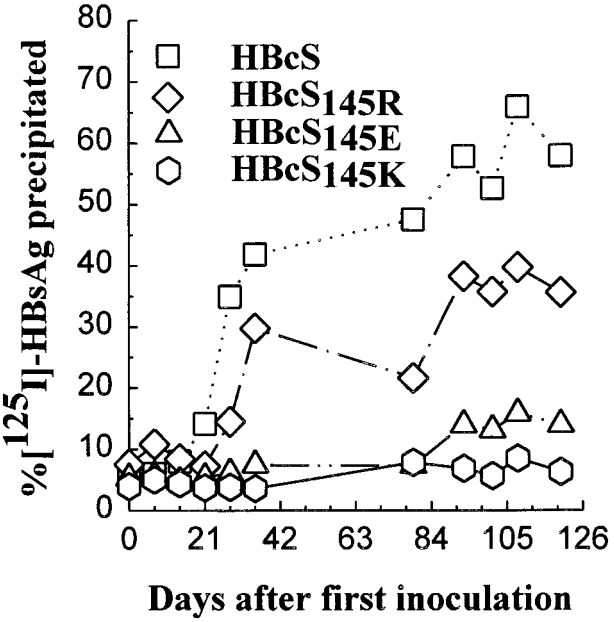

Fig. 5. Anti-HBs production in four rabbits immunized with HBcS or its Gly_{145} mutants, as described in the legend to Figure 4. Serum samples were tested for anti-HBs antibody by the DARIP assay using [^{125}I]-labelled HBsAg (*adw*) as the reagent. The amounts of anti-HBs antibody present in sera were expressed as a percentage of [^{125}I]-antigen precipitated by donkey anti-rabbit serum compared to total [^{125}I]-HBsAg in the reaction.

polyclonal probes would be expected to contain antibodies against a number of different B-cell epitopes in the $HBs_{(111–156)}$ sequence.

Confirmation of the presentation of an immunologically active HBsAg domain by $HBcS_{(111–156)}$ was provided by the induction of anti-HBs in rabbits [Stahl and Murray, 1989], and some of the fusion proteins carrying the mutated epitope exhibited similar properties (Fig. 5).

In the AUSRIA test, HBsAg is sandwiched between two anti-HBs antibodies, one immobilized on the solid phase and the other labelled with ^{125}I as the probe. Even at high concentrations (100–500 μg), the HBcAg fusion proteins carrying the $HBs_{(111–156)}$ sequence reacted poorly with anti-HBs antibody in this assay, possibly because the conformation of the $HBs_{(111–156)}$ sequence in the fusion protein context differs from its native form and its epitopes are thereby hindered from reacting efficiently with anti-HBs in either solid or liquid phases. The configuration of the IRMA was therefore changed; human anti-HBc was substituted for anti-HBs as the solid phase-bound antibody and rabbit anti-HBs followed by [^{125}I]-labelled donkey anti-rabbit IgG was used as the probe. The HBcS fusion particles then displayed HBsAg reactivity in a concentration-dependent manner (Fig. 1B), and the assay showed that HBcAg and HBsAg reactivities were manifest in the same molecule or particle. The levels of the fusion proteins required for detection of HBsAg reactivity were about 1,000-fold higher (by weight, 300-fold on a molar basis) than those used in an equivalent assay for HBcAg, but, and importantly, only fusion proteins displaying both HBcAg and HBsAg antigenicities could be detected; at similar concentrations, HBc fusions carrying preS1 or preS2 segments showed no reactivity with anti-HBs.

The *a* determinant contained within aa residues 122–137 and 139–147 of the S protein is believed to be conformational [Dreesman et al., 1982; Brown et al., 1984]. Peptides derived from this region of HBsAg displayed much weaker antigenicity than native, intact HBsAg, and the HBs antigenicity of the HBcAg fusion proteins is also weak compared with that of native HBsAg. The HBcAg fusion proteins, carrying either wild-type or a mutant $HBs_{(111–156)}$ segment, also reacted poorly with anti-*a* monoclonal antibodies that bind to cyclic peptides made from aa 124–137 or 139–147 of HBsAg (data not shown).

Although their reactions with antibodies to HBsAg were weak, the fusion proteins carrying the $HBs_{(111–156)}$ fragment induced antibodies that reacted effectively with native HBsAg. In rabbits, the hybrid HBcAg particles were also immunologically efficient in terms of T-cell activation as measured by lymphocyte-proliferative responses in vitro. These strong responses (Figs. 2, 3) are attributable to the HBcAg component of the fusion proteins because similar strong HBcAg-specific responses have been observed in mice [Milich et al., 1987b] and humans [Ferrari et al., 1990], whereas HBsAg induces only weak and inconsistent stimulatory

responses. In patients with acute hepatitis B, the appearance of a detectable T-cell response to HBcAg was correlated with the clearance of HBsAg from the serum [Ferrari et al., 1990], suggesting that amplification of the anti-HBs B-cell response might occur via a direct interaction of HBcAg-specific T cells with HBsAg-specific B cells [Milich et al., 1987a]. Also, HBsAg-specific T- and B-cell responses could be augmented by the release of antigen-nonspecific lymphokines by activated HBcAg-specific T cells, as was observed in B10.S mice, a nonresponding strain to HBsAg, immunized simultaneously with HBcAg and HBsAg [Shiau, 1993].

While all HBcAg fusion proteins eliciting high levels of anti-HBc were correlated with high HBcAg-specific T-cell responses, the anti-HBs responses to HBcS bearing different substitutions at aa 145 of HBsAg differed. HBcS and $HBcS_{145R}$ induced antibodies that cross-reacted with native HBsAg, but $HBcS_{145E}$ and especially $HBcS_{145K}$ induced only minimal levels of such antibodies. Some variation in anti-HBs production was observed between different rabbits, which is to be expected in outbred animals, but the levels of anti-HBs induced in rabbits and in BALB/c mice by HBcAg fusion proteins carrying the $HBs_{(111–156)}$ fragment were lower than those elicited by native HBsAg (data not shown), in accord with earlier reports that peptides derived from the 110–150 region of HBsAg and conjugated to a carrier protein induced a lower level of anti-HBs than did native HBsAg [Bhatnagar et al., 1982; Neurath et al., 1982; Prince et al., 1982].

It is particularly interesting that the response to $HBcS_{145R}$ was comparable to that induced by HBcS in terms of antibodies that cross-react with wild-type or native HBsAg. This contrasts with the inability of HBsAg bearing the 145R mutation to cross-react with antibodies to native HBsAg [Bruce and Murray, 1995] and with the anti-HBs response in mice to the same variant of HBsAg (synthesised in yeast), where the second inoculation resulted in a high antibody titre to the homologous (i.e., mutant) antigen but a 100-fold lower titre of antibodies to wild-type HBsAg than that induced by the native antigen [Waters et al., 1992b].

Inclusion of preS1 and preS2 epitopes in the HBcS or $HBcS_{145R}$ fusion proteins further enhanced their immunogenicity with regard to anti-HBs antibodies [Shiau, 1993]. Inclusion of multiple epitopes from these regions in fusions to HBcAg may therefore offer a useful approach to the generation of neutralising antibodies against HBV and the possibility of protection against variants that have yet to be discovered or, indeed, to arise. Alternatively, a combination of different sequences of the S epitope, either on the same particle or in a mixture of particles, may achieve the same end. In principle, vaccines based upon HBcAg carrying the HBV envelope epitopes have the advantage not only of inducing HBcAg-specific cytotoxic T cells against wild-type and mutant viruses but also of possibly priming T helper cells that can augment anti-HBs production following subsequent exposure to HBV.

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